Fungal Biology 123 (2019) 209–217

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Dominant effects of *gat1* mutations on the ligninolytic activity of the white-rot fungus *Pleurotus ostreatus*

Takehito Nakazawa*, Ryota Morimoto, Hongli Wu, Rina Kodera, Masahiro Sakamoto, Yoichi Honda

Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

ARTICLE INFO

Article history:
Received 6 September 2018
Received in revised form
8 December 2018
Accepted 20 December 2018
Available online 27 December 2018

Corresponding Editor: Nabla Kennedy

Keywords:
Basidiomycete
Fruiting
Lignin
Mushroom
Agaricomycete
Wood decay

ABSTRACT

In nature, white-rot fungi efficiently degrade lignin present in wood biomass. Elucidation of molecular mechanisms underlying wood lignin biodegradation by white-rot fungi would contribute to the development of efficient and ecofriendly methods of producing valuable chemical products from wood biomass. Here, using forward genetics approach, we demonstrate that the mutant of a putative transcription factor gene, *gat1-1*, significantly decreases the ligninolytic activity of the white-rot fungus *Pleurotus ostreatus*, when grown on beech wood sawdust medium. We also show that this phenotype is dominant. In *Schizophyllum commune*, Gat1 was previously shown to be involved in fruiting body development. In this study, we reveal that the mutations in *gat1* gene cause defects in fruiting body development in *P. ostreatus*. Unlike the previously reported recessive gene mutations that decrease the ligninolytic activity of *P. ostreatus*, the *gat1-1* mutation and Δ*gat1* are dominant and would thus be useful for future studies on the functional role of the orthologs in other white-rot fungi.

1. Introduction

Cellulose, hemicellulose, and lignin are the three major components of wood biomass. Cellulose is a linear β-1,4-glucan that forms crystalline cellulose (Kolpak and Blackwell, 1976). Hemicellulose is a branched and heterogeneous polysaccharide with shorter chains than cellulose. Generally, glucuronoxylan and galactoglucomannan are the primary hemicelluloses contained in hardwoods and softwoods, respectively (Gírio et al., 2010). Unlike cellulose and hemicellulose, lignin is an aromatic heteropolymer consisting of phenylpropanoid unit(s) (Boerjan et al., 2003). Lignin monomers and some sugars composing hemicellulose are linked by covalent bonds (Watanabe and Koshijima, 1988; Watanabe et al., 1989; Nishimura et al., 2018). Cellulose and some hemicelluloses are also linked by hydrogen bonds (Zhang et al., 2011, 2015). Thus, wood biomass is complex and exhibits high resistance to biodegradation.

In nature, it is generally considered that the aforementioned recalcitrant wood biomass are primarily degraded by wood-decaying fungi, most of which belong to Agaricomycetes. The wood-decaying agaricomycetes are typically divided into two types: white-rot and brown-rot fungi. White-rot fungi decompose lignin more efficiently than polysaccharides, and it is generally thought that they play an important role in the global carbon cycle. On the other hand, brown-rot fungi efficiently decompose crystalline cellulose via non-enzymatic mechanisms (Arantes and Goodell, 2014) and are almost incapable of decomposing lignin, besides modifying chemical structure of lignin (Jin et al., 1990).

Catalytic properties of various enzymes that may be involved in the decomposition of the three major components have been analyzed (Rytioja et al., 2014; Pollegioni et al., 2015). Lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs) are generally considered key enzymes that modify/depolymerize lignin, although it has been suggested that other enzymes are also involved in lignin degradation (Kersten and Cullen, 2014; Riley et al., 2014). Recent genomic/transcriptome/proteome studies have shown that gene number and the expression pattern of putative ligninolytic, hemicellulolytic, and cellulolytic genes/enzymes differ among some white-rot fungi in the presence of wood biomass (Fernández-Fueyo et al., 2012; Hori et al., 2014; Riley et al., 2014), thereby providing insights into the diversity of strategies for wood decomposition.

* Corresponding author. Graduate School of Agriculture, Kyoto University, Oiwakecho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan. Fax: +81 75 753 6471.
E-mail address: tnakazaw@kais.kyoto-u.ac.jp (T. Nakazawa).

https://doi.org/10.1016/j.funbio.2018.12.007
1878-6146/

Pleurotus ostreatus, the oyster mushroom, is one of the white-rot fungi frequently used for studies on ligninolytic enzymes (Kamitsuji et al., 2004, 2005; Salame et al., 2010). Six *mnp* (*mnp1–6*) and three *vp* (*vp1–3*) genes are predicted in the genome of *P. ostreatus* (Knop et al., 2015). Engineering of MnPs derived from this fungus has also been reported (Tsukihara et al., 2008; Fernández-Fueyo et al., 2014). Additionally, this fungus is useful for molecular genetic studies owing to the efficient gene targeting system that was established by creating a *ku80* knockout strain (Salame et al., 2012). Single-gene disruptants for some *mnp*/*vp* genes were isolated and their phenotypes were analyzed (Salame et al., 2013, 2014; Nakazawa et al., 2017b).

Mechanisms underlying transcriptional regulation of genes encoding ligninolytic enzyme have been investigated in some white-rot fungi when grown on certain liquid medium (Alvarez et al., 2009; Toyokawa et al., 2016; Feldman et al., 2017). Sakamoto et al. (2010) and Suetomi et al. (2015) demonstrated the differences in the effect of a calmodulin inhibitor, W7, on extracellular Mn^{2+}-dependent peroxidase activity between two white-rot fungi, *P. ostreatus* and *Phanerochaete chrysosporium*, thereby suggesting a diversity in regulatory mechanisms. Nakazawa et al. (2017a; 2017b) identified three gene mutations that cause defects in decolorization of Remazol Brilliant Blue R (RBBR) and Orange II, which are frequently used to examine the ligninolytic activity of *P. ostreatus* (Vyas and Molitoris, 1995; Salame et al., 2010), through an efficient forward genetic approach. The *wtr1* gene encodes a putative Agaricomycete-specific DNA-binding transcription factor (Nakazawa et al., 2017a). The *wtr1-1* mutation, which misses a fungal-specific transcription factor domain, caused defects in decolorization of RBBR and Orange II, but not wood lignin degradation. However, targeted disruption of *wtr1* causes defects in the ability to degrade wood lignin to some degree (Nakazawa et al., 2017a). In contrast to *wtr1*, targeted disruptions of two other genes, *chd1* encoding a putative chromatin remodeler and *pex1* encoding a peroxisome biogenesis factor, were shown to cause significant defects in wood lignin degradation in our previous studies (Nakazawa et al., 2017a, 2017b). However, the mechanisms that underlying transcriptional regulation of lignin degradation in *P. ostreatus* and other white-rot fungi still remain unclear.

Here, we identified a mutant of *P. ostreatus* defective in ligninolysis on sawdust media. Unlike the other mutations identified in the previous studies, the phenotype of this mutant was dominant. It was also revealed that this mutant is also dominant defective in fruiting. We found that the gene responsible for the dominant mutant phenotypes of this strain is homologous to the *Schizophyllum commune gat1* gene that has been shown to be involved in its fruiting body development (Ohm et al., 2011).

2. Materials and methods

2.1. Strains, culture conditions, and genetic techniques of P. ostreatus

The *P. ostreatus* strains used in this study are listed in Table S1. Yeast and malt extract with glucose (YMG) medium (Rao and Niederpruem, 1969) solidified with 2 % (w/v) agar in 9 cm Petri dishes was used for routine cultures. YMG supplemented with 27 μM $MnSO_4$ and 64 μM RBBR (YMGMnR) was used to examine the ability of each *P. ostreatus* strain to decolor RBBR. Glucose-peptone (GP; Salame et al., 2012) liquid medium and GP supplemented with 27 μM $MnSO_4$ (GPMn) were used to measure extracellular enzyme activity. GP and GPMn are often used to examine Mn^{2+}-dependent peroxidase activity in PC9-derived strains (Salame et al., 2012, 2013).

Beech wood (*Fagus crenata*) sawdust media A (4.1 g sawdust, 0.3 g wheat bran, and 10.6 ml H_2O) and B (1.9 g sawdust, 0.1 g wheat bran, and 6.0 ml H_2O) were used to examine the ability of each *P. ostreatus* strain to degrade wood lignin in this study. Sawdust media C (1.9 g extracted sawdust, 0.1 g extracted wheat bran, and 6.0 ml H_2O) and D (1.6 g extracted sawdust, 0.4 g extracted wheat bran, and 6.0 ml H_2O) were also used to measure extracellular enzyme activity and to isolate total RNA. In this study, wheat bran was mixed with beech wood sawdust to enhance the production of ligninolytic enzymes (Pickard et al., 1999; Tsukihara et al., 2006). 20 mM Uridine and 0.18 mM uracil were supplemented into sawdust media C and D when strain 20b/1 and *chd1*/*pex1* disruptants derived from 20b/1 [J3-3d#1, D14d#1 and D14d#2 (Table S1)] were grown. Sawdust and wheat bran used in this study were purchased from Shinkoen (Gifu, Japan) and Nisshin Seifun (Tokyo, Japan), respectively. They included moisture because they were simply stored at 4 °C or room temperature. In this study, sawdust used for the preparation of sawdust media A and B was subject to size-fractionation (250–500 μm). Extractives present in sawdust and wheat bran were removed using toluene and ethanol (2:1 v/v) for 1 h at 80 °C for four times, when preparing sawdust media C and D. The cultures were maintained at 28 °C under continuous darkness, unless stated otherwise.

UV mutagenesis, crosses, and fruiting of *P. ostreatus* were performed as described by Nakazawa et al. (2016), Inada et al. (2001), and Nakazawa et al. (2016), respectively. The transformation of *P. ostreatus* strains was performed using protoplasts prepared from mycelial cells as described by Salame et al. (2012), with some modifications (Nakazawa et al., 2016).

2.2. Assay for extracellular enzyme activity

Each *P. ostreatus* strain was grown stationary on GP and GPMn liquid media as described by Nakazawa et al. (2016). Measurement of extracellular enzyme activity [2-Methoxyphenol (guaiacol) oxidation] was performed as described by Kamitsuji et al. (2004) with some modifications (Nakazawa et al., 2017a). Extracellular enzyme activity of each strain when grown on sawdust medium C was measured as described by Nakazawa et al. (2017b). In this study, one unit of activity for guaiacol oxidation is defined as the amount of enzyme that increases the absorbance at 465 nm by 1.0 per min in this study.

2.3. Quantification of Klason lignin, xylose, and α-cellulose

Each *P. ostreatus* strain (Table S1) was grown on sawdust medium A for 28 d (PC9 and UVRM22), on sawdust medium B for 20 d (20b, gat1d#1, and gat1d#2), or on sawdust medium B for 13 d (dikaryon strains), followed by the quantification of the residual amount of Klason lignin (acid-insoluble) contained in each sawdust medium as described by Nakazawa et al. (2016). Briefly, sawdust media were first subject to solvent extraction with toluene and ethanol (2:1, v:v) for 1 h twice at room temperature. Extracted sawdust media were then dried in an oven (50 °C) for at least for 24 h, and the weight of each sample was measured. About 0.5 g of each dried sawdust medium was then hydrolyzed by H_2SO_4 as described by Ritter et al. (1932). The resulting acid-insoluble lignin was collected by suction filtration and dried at 50 °C for 24 h, followed by the measurement and calculation of the weight of Klason lignin. Quantification of the residual amount of α-cellulose and xylose contained in sawdust medium was performed as described by Nakazawa et al. (2017b).

2.4. Whole-genome resequencing and bioinformatics

Extraction of genomic DNA, whole-genome resequencing, alignment to the genome of strain PC9, and browsing of visualized mutations were performed as described by Nakazawa et al. (2017a).

2.5. Introducing the gat1-1 mutation into PC9 and creating gat1 disruptants from 20b

To introduce the *gat1-1* mutation into the wild-type strain PC9 (Table S1), cotransformation was performed (Marmeisse et al., 1992; Irie et al., 2001; Weber et al., 2005; Nakazawa et al., 2017a). A genomic fragment containing the gene that corresponds to Protein ID 83134 [*gat1*; Scaffold_2: 673565–678152 in the genome database of strain PC9 (In: https://genome.jgi.doe.gov/PleosPC9_1/PleosPC9_1.home.html)] was amplified by polymerase chain reaction (PCR) from UVRM22 using primer pair RM1/RM2 (Table S2). This fragment was then introduced into PC9 concomitantly with the pTN24-1 plasmid carrying the hygromycin-B-resistance gene (Nakazawa et al., 2017b). As a control, we also introduced plasmids pTN25 and pTN25-2, which expressed mCherry- and EGFP-harboring peroxisomal target signal 1 (Nakazawa et al., 2017b), respectively, into PC9 along with pTN24-1. After 10–20 d of transformation, hygromycin-B-resistant colonies appeared on the regeneration medium (Salame et al., 2012). They were transferred onto a YMG agar plate containing 100 μg/ml (w/v) hygromycin B. After this passage, the ability of each hygromycin-B-resistant transformant to decolor the RBBR present in YMGMnR agar plates was examined.

The plasmid for *gat1* disruption was created as described by Nakazawa and Honda (2015). Briefly, a genomic fragment amplified using primer pair RM1/RM2 was cloned into pBluescript II KS + digested with *Eco*RV. Inverse PCR was performed with the resulting plasmid as a template and the primer pair RM51/RM52. A DNA fragment containing the hygromycin-B-resistance gene was also amplified using pTN24-1 as a template and the primer pair TN400/M13R. The resulting two DNA fragments were fused using the Geneart Seamless Cloning and Assembly kit (Life Technologies, CA, USA) to yield a plasmid containing the *gat1*-disrupting cassette. We then introduced this plasmid into strain 20b to obtain *gat1* gene knockouts, namely gat1d#1–4 (Table S1; Fig. S4).

2.6. RNA-sequencing (RNA-seq) and quantitative reverse transcription-PCR (qRT-PCR)

The extraction of total RNA prepared from each strain grown on sawdust medium C or D was carried out as described by Nakazawa et al. (2017a). RNA-seq analysis was carried out as described by Nakazawa et al. (2017b).

For qRT-PCR, the obtained RNA samples were reverse-transcribed using PrimeScript RT kit (Takara, Shiga, Japan), followed by quantitative PCR. In this study, GoTaq qPCR Master Mix (Promega, WI, USA), and the Thermal Cycler Dice Real Time System Lite (Takara, Shiga, Japan) were used. Quantification was carried out as described by Pfaffl (2001). Primer pairs used for the amplification of cDNA fragments of *β-tubulin*, *vp2*, and *vp3* genes are indicated in Salame et al. (2013). Amplification efficiencies for each of these primer pairs were 96.3 %, 93.0 %, and 88.3 %, respectively.

3. Results

3.1. Isolation and phenotypic analysis of a P. ostreatus mutant UVRM22

We isolated a *P. ostreatus* mutant defective in decolorization of RBBR, namely UVRM22, after UV mutagenesis of wild-type strain PC9 (Table S1). RBBR is frequently used to examine the ligninolytic activity of this fungus. PC9, but not UVRM22, decolored RBBR present in YMGMnR agar medium at 8 d after inoculation (Fig. S1A; PC9 usually decolors RBBR within 5–7 d). This mutant also produced almost no extracellular enzymes that can oxidize guaiacol in the presence of manganese when grown on sawdust medium C as well as GP and GPMn liquid media when compared with the wild-type strain PC9 (Fig. 1A and B; strains PC9 and UVRM22 take about 10 d to fully cover sawdust media used in this study). Also, PC9, but not UVRM22, whitened sawdust medium C at 13 d after inoculation (Fig. S1B). PC9 and UVRM22 were then grown on sawdust medium A for 28 d to examine the ability of these strains to degrade wood lignin. As shown in “Lignin loss” indicated in Table 1, PC9 degraded lignin contained in sawdust medium A better than UVRM22. “Composition change” indicated in Table 1 reflects the different ability of each strain to whiten the sawdust medium, suggesting that UVRM22 is less effective in wood lignin degradation.

3.2. UVRM22 is a dominant mutant defective in ligninolysis and fruiting

When UVRM22 was mated with a wild-type monokaryon, PC15 (Table S1; this strain forms dikaryon when mated with PC9), the resulting dikaryon did not efficiently decolor the dye present in YMGMnR agar medium and did not whiten sawdust medium C (Fig. S1A and B). PC9 × PC15 dikaryon decolored RBBR present in YMGMnR agar medium and whitened sawdust medium C much more than UVRM22 × PC15 one (Fig. S1A and B). These results suggest that the less ligninolytic phenotype of UVRM22 is dominant.

To identify the pattern of inheritance of the phenotypic traits of UVRM22, we attempted to produce fruiting bodies of UVRM22 × PC15 dikaryon to isolate F_1 progeny. The PC9 × PC15 dikaryon usually forms primordia about 3–5 d after induction of fruiting (a temperature downshift from 28 °C to 18 °C, under a 12 h light/12 h dark cycle), and fruiting bodies within 10 d after induction (Fig. 1C; at 8 d after induction). However, we found that UVRM22 × PC15 did not produce primordia and fruiting bodies when grown on the sawdust medium used for fruiting (Fig. 1C). No primordium/fruiting body was formed on the sawdust medium growing UVRM22 × PC15 even at 14 d after induction (data not shown). Moreover, the sawdust medium used for fruiting was whitened by PC9 × PC15 dikaryon, but not by UVRM22 × PC15 dikaryon in this study (Fig. S2). These results suggest that the mutant phenotypes (defects in ligninolysis and fruiting) of the isolated strain are caused by dominant mutation(s).

3.3. Identification of the mutant gene responsible for defects of UVRM22 in RBBR decolorization

The fact that UVRM22 × PC15 dikaryon is unable to form fruiting bodies precluded us from genetic analysis of F_1 progeny from UVRM22 × PC15 dikaryon. Therefore, we decided to identify the gene(s) responsible for defects of UVRM22 strain in RBBR decolorization, without genetic analysis. Whole-genomic resequencing identified 20 mutations (nucleotide substitutions) introduced into the genome of UVRM22 based on the survey of Scaffold 1–36 of the genome database of strain PC9. Fortunately, this number is much smaller than those usually identified in genomes of the other mutants shown in our previous studies. Among these mutations, only five seemed to cause amino acid substitutions (Table S3), suggesting that one of the five mutations is likely responsible for the dominant mutant phenotype of strain UVRM22. To confirm which mutation(s) is responsible for the mutant phenotype, we first introduced a genomic fragment containing the mutant gene

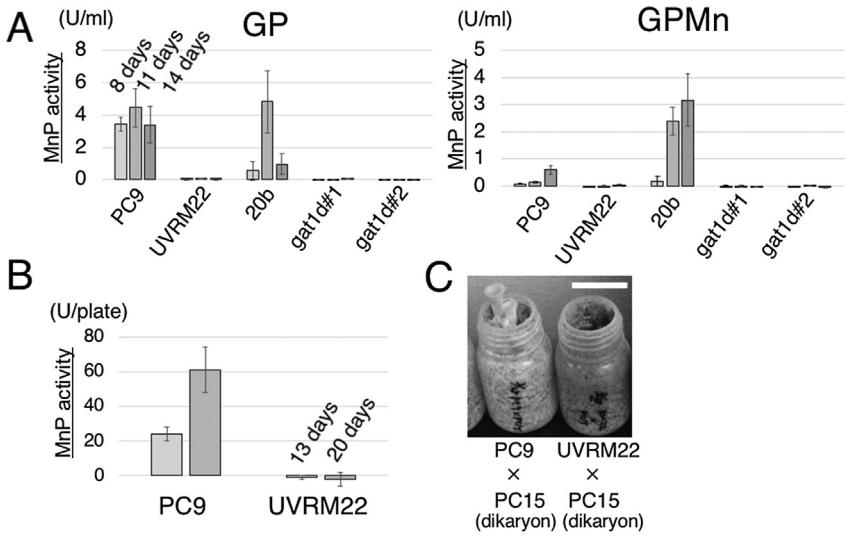

Fig. 1. Extracellular Mn^{2+}-dependent peroxidase activity and fruiting body development on sawdust medium of each *P. ostreatus* strain. (A) Extracellular Mn^{2+}-dependent peroxidase activity of indicated strains when grown on GP and GPMn liquid media for 8, 11 and 14 d. The error bars represent standard deviation (n = 3). One unit (U) of activity for guaiacol oxidation is defined as the amount of enzyme that increases the absorbance at 465 nm by 1.0 per min. (B) Mn^{2+}-dependent peroxidase activity of extracellular enzymes produced by the indicated strains grown on sawdust medium C for 13 or 20 d. Error bars represent standard deviation (n = 3). For descriptions regarding the defects of UVRM22 (and UVRM22 × PC15 dikaryon) in RBBR decolorization and wood lignin degradation, please see Fig. S1 and Table 1, respectively. (C) Fruiting body development of indicated dikaryon strains when grown on sawdust medium (8 d after induction). Scale bar represents 2.5 cm. A color picture is provided in Fig. S2.

Table 1
The degradation of wood lignin by each *P. ostreatus* strain under each culture condition.

Strain	Sawdust medium	Culture days	Lignin loss[a] [%]	Composition change[b] [%]	No. of replications
PC9	A	28 d	30.5 ± 1.8	−4.6 ± 0.3	3
UVRM22	A	28 d	−0.8 ± 1.1	+ 0.8 ± 0.4	3
20b	B	20 d	19.2 ± 1.1	−1.7 ± 0.2	3
gat1d#1	B	20 d	0.9 ± 5.1	+1.5 ± 1.2	3
gat1d#2	B	20 d	−1.1 ± 1.3	+2.1 ± 0.3	3
20b × PC15	B	13 d	21.6	−1.2	1
gat1d#1 × PC15	B	13 d	0.5	+ 1.4	1
gat1d#2 × PC15	B	13 d	4.3	+ 1.1	1
gat1d#3 × PC15	B	13 d	−1.8	+ 1.8	1
gat1d#4 × PC15	B	13 d	5.1	+ 1.2	1

[a] Decrease in the weight of Klason lignin per plate compared with the No-fungus control plate (0.8310 g/plate for sawdust A and 0.4091 g/plate for B).
[b] Change in the composition of lignin contained in the solvent-treated residual sawdust medium compared to the No-fungus control (21.2 % w/w for sawdust A and 22.2 % w/w for B).

encoding a putative transcription factor corresponding to Protein ID 83134 (https://genome.jgi.doe.gov/cgi-bin/dispGeneModel?db=PleosPC9_1&id=83134) derived from UVRM22 (Table S3), concomitantly with plasmid pTN24-1 carrying the hygromycin-B-resistance gene into the wild-type strain PC9. Out of the obtained 54 hygromycin-B-resistant transformants, 12 were defective in RBBR decolorization. As a control, we also introduced plasmids pTN25 and pTN25-2, which do not contain the mutant gene, into PC9 along with pTN24-1. None of the total 27 control transformants obtained were defective in decolorization of RBBR. These results suggest that the mutation in a gene corresponding to 83134 causes the dominant defect at least in RBBR decolorization by *P. ostreatus*. In this study, we did not introduce other four mutant genes into PC9 to check whether these mutations are not responsible for the mutant phenotypes of UVRM22.

3.4. *The responsible gene is homologous to the* S. commune gat1 *gene*

The gene corresponding to 83134 encodes a putative protein harboring a GATA-type zinc finger DNA-binding motif (Fig. 2A). The exon-intron prediction based on our RNA-seq data of strains PC9, 20b, and 20b/1 (when grown on sawdust medium C or D for 13 d) was not identical to annotation in the JGI genome database (Fig. S3). To evaluate which prediction is correct, we performed RT-PCR using primer pair TN752/TN753, followed by DNA sequencing. These results indicated that the exon-intron prediction based on our RNA-seq data is correct. We thereby registered the sequenced cDNA (GenBank/EMBL/DDBJ Accession ID: LC383432). The protein encoded by this gene (665 amino acids in length) is conserved among Agaricomycetes, including a saprophytic agaricale *Coprinopsis cinerea* (XP_001837549; *E*-value, 5e-71), an agaricale *S. commune* Gat1 (Ohm et al., 2011; XP_003036589; *E*-value, 4e-52), a selective white-rot polyporale *Gelatoporia subvermispora* (EMD40513; *E*-value, 1e-61), and a brown-rot polyporale *Fomitopsis pinicola* (EPS98753, *E*-value, 2e-51), but not those from *Cryptococcus neoformans*, *Ustilago maydis*, and Ascomycetes. In particular, approximately 60 amino acids around the zinc finger motif is almost completely conserved among putative proteins from Agaricomycetes. Therefore, we designated this newly identified gene as *P. ostreatus gat1*. The mutation introduced into the genome of strain UVRM22 causes an amino acid substitution within the zinc finger motif of the Gat1 protein (S233F; Fig. 2A), suggesting malfunction of the DNA-binding motif. We designated this mutation as *gat1-1* (Table S1).

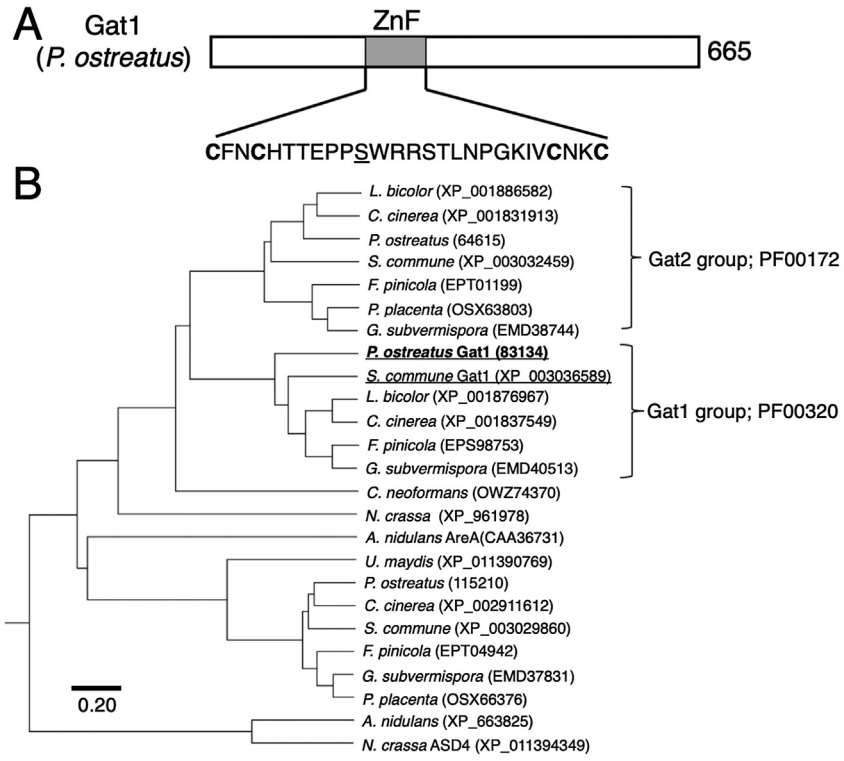

Fig. 2. Primary structure analysis and phylogeny of Gat1 proteins. (A) Structural properties and amino acid sequence of zinc finger motif of the *Po*Gat1 protein. The underlined serine 233 is altered into proline in mutant strain UVRM22. (B) Phylogenetic analysis of fungal proteins that exhibit high similarity to Gat1 drawn using the Unweighted Pair Group Method with Arithmetic mean. Protein IDs for *P. ostreatus* proteins are from the JGI genome database. Accession numbers for putative proteins from other fungal species were obtained from NCBI database. Scale bar indicates the bootstrap value.

3.5. *Phylogenetic analysis revealed another putative Agaricomycete-specific DNA-binding transcription factor similar to Gat1*

Fig. 2B shows a phylogenetic tree of putative proteins that exhibit high similarity to Gat1. This phylogenetic tree revealed a gene encoding another putative transcription factor exhibiting high similarity to Gat1 and is conserved among Agaricomycetes. We designated this gene as *gat2*. It was also shown that *Aspergillus nidulans* AreA (Fig. 2B), which is a transcriptional regulator mediating nitrogen metabolite repression in Aspergilli (Kudla et al., 1990; Monahan et al., 2006), is relatively similar to Gat1 and Gat2 proteins. This finding might raise the possibility that Gat1 (or Gat2) is not an Agaricomycete-specific transcription factor, but a homolog of AreA. However, this possibility is excluded by the fact that another gene encoding a putative protein much more similar to AreA is present in the genome of *P. ostreatus* as well as some agaricomycete species (Fig. 2B).

3.6. *Effects of targeted disruption of* gat1 *on the ability to degrade wood biomass and fruiting of* P. ostreatus

Four *gat1* disruptants, gat1d#1, gat1d#2, gat1d#3, and gat1d#4 (Table S1 and Fig. S4), were generated from strain 20b (a *ku80* disruptant; Table S1). Strain 20b, but not gat1d#1 and gat1d#2, decolored RBBR present in YMGMnR agar medium at 9 d after inoculation (Fig. S5A) and produced extracellular Mn^{2+}-dependent peroxidase when grown on sawdust medium C as well as on GP and GPMn liquid media (Figs. 1A and 3A). Strains gat1d#3 and gat1d#4 also did not decolor RBBR when they were grown on YMGMnR agar medium for 11 d (data not shown). As shown in Fig. 3B, hyphal growth rates of *gat1* disruptants were slightly slower than that of its parental strain 20b. Strains 20b, gat1d#1, and gat1d#2 were also grown on sawdust medium B for 20 d to examine the effects of *gat1* disruption on the ability to degrade wood lignin. It was observed that 20b degrades lignin contained in sawdust medium B much more than the two *gat1* disruptants ("Lignin loss" indicated in Table 1). The cultivation period for this experiment is shorter compared to growth of PC9 and UVRM22 on sawdust medium A (28 d) because the amount of sawdust and wheat bran contained in sawdust medium B (2.0 g) is less than that in sawdust medium A (4.4 g).

We also examined the effects of *gat1* disruption on the ability to degrade polysaccharides contained in sawdust medium B. Decreases in the amount of xylose (Table 2) and α-cellulose (Table 3) reflect xylanolytic (hemicellulolytic) and cellulolytic activities, respectively. As shown in Tables 2 and 3, a significant difference was not observed when compared with parental strain 20b. These results suggest that *gat1* disruption decreases defects in ligninolysis, but not cellulolysis and xylanolysis.

To examine whether the disruption of *gat1* also causes dominant defects in ligninolysis and fruiting, the four *gat1* disruptants and 20b were mated with PC15. The resulting 20b × PC15 dikaryon decolored RBBR present in YMGMnR agar medium much more than gat1d#1–4 × PC15 dikaryons (Fig. S5A). 20b × PC15, but not gat1d#1–4 × PC15, formed primordia and fruiting bodies (Fig. 3C; at 7 d after induction) and whitened sawdust medium (Figs. S2 and S5B). 20b × PC15 and gat1d#1–4 × PC15 dikaryons were also grown on sawdust medium B for 13 d to examine the ability of each dikaryon strain to degrade wood lignin. Dikaryon strains generally grow faster than monokaryons (The dikaryon strains used in this study fully covered the glass plate within 10 d post inoculation). Therefore, we changed the cultivation period for this experiment. As shown in "Lignin loss" of Table 1, the control dikaryon strain

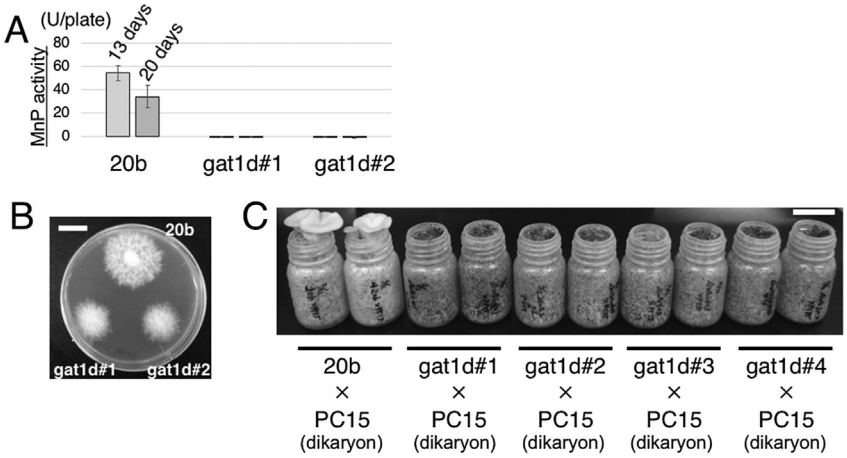

Fig. 3. Effects of *gat1* disruption on extracellular Mn^{2+}-dependent peroxidase activity and fruiting body development. (A) Mn^{2+}-dependent peroxidase activity of extracellular enzymes produced by the indicated strains grown on sawdust medium C for 13 or 20 d. Error bars represent standard deviation (n = 3). For descriptions regarding the defects of Δ*gat1* strains (and Δ*Pogat1* × PC15 dikaryons) in RBBR decolorization and wood lignin degradation, please see Fig. S5A and Table 1, respectively. (B) Hyphal growth rate of the indicated three strains when grown on YMG agar plate (7 d). Scale bar represents 2 cm. (C) Fruiting body development of indicated dikaryon strains when grown on sawdust medium (7 d after induction). Scale bar represents 2.5 cm. A color picture is provided in Fig. S2.

Table 2
The biodegradation of the polysaccharide composed of xylose by each *P. ostreatus* strain under each culture condition.

Strain	Sawdust medium	Culture days	Xylose loss[a] [%]	No. of replications
20b	B	20 d	10.6 ± 7.2	3
gat1d#1	B	20 d	8.7 ± 5.0	3
gat1d#2	B	20 d	8.7 ± 6.1	3

[a] Decrease in the amount of xylose per plate compared with the No-fungus control plate onto which the *P. ostreatus* strain was not inoculated (0.4698 g/plate for the sawdust medium B).

Table 3
The changes in the amount of α-cellulose after the cultivation of each *P. ostreatus* strain under each culture condition.

Strain	Sawdust medium	Culture days	α-cellulose loss[a] [%]	No. of replications
20b	B	20 d	12.9 ± 0.8	3
gat1d#1	B	20 d	10.4 ± 1.7	3
gat1d#2	B	20 d	13.9 ± 1.7	3

[a] Decrease in the amount of α-cellulose per plate compared with the No-fungus control plate (0.8168 g/plate) onto which the *P. ostreatus* strain was not inoculated.

(20b × PC15: *gat1*$^+$ × *gat1*$^+$) degraded lignin contained in sawdust medium B much more than the Δ*gat1*×*gat1*$^+$ dikaryon strains. Fig. S5B and "Composition change" of Table 1 also suggested that the Δ*gat1*×*gat1*$^+$ strains are defective in white rot. Thus, targeted disruption of *gat1* gene causes mutant phenotypes similar to those of UVRM22, such as dominant defects in ligninolysis and fruiting of *P. ostreatus*.

3.7. *Effects of* gat1, wtr1, pex1, *and* chd1 *disruptions on* vp2 *and* vp3 *transcript levels*

It was shown that *vp2* and *vp3* transcripts accumulate predominantly among genes encoding MnPs and VPs when strain 20b/1, a *pyrG* mutant derived from 20b (Nakazawa et al., 2016; Table S1), was grown on sawdust medium D supplemented with uridine and uracil for 13 d (Nakazawa et al., 2017b). To confirm if *vp2* and *vp3* transcripts also accumulate abundantly in PC9 and 20b, we performed RNA-seq analysis (Table S4). It was shown that *vp2* and *vp3* transcripts also accumulate predominantly when PC9 and 20b are grown on sawdust medium C for 13 d. These results suggest that at least *vp2* and *vp3* play an important role in lignin degradation on the beech wood sawdust used in this study. Thus, we examined the effects of *gat1* disruption on accumulation of *vp2* and *vp3* transcripts by qRT-PCR when grown on sawdust medium C for 13 d in this study.

Here, we examined the effects of *wtr1*, *chd1*, *pex1* disruptions as well as *gat1* disruption on the accumulation of *vp2* and *vp3* transcripts because we had performed semi-quantitative RT-PCR using *wtr1* and *pex1* disruptants and the mutant strain UVJ3-3 (the *chd1-1* mutant; Nakazawa et al., 2017a) in our previous studies (Nakazawa et al., 2017a, 2017b). 20b/1 and *pex1* disruptants were grown on sawdust medium D supplemented with uridine and uracil because *pex1* disruptants do not grow well on sawdust medium C, which contains smaller amounts of wheat bran (Nakazawa et al., 2017b). As shown in Fig. 4, *gat1*, *chd1*, and *pex1* disruptions reduced *vp2* transcript accumulation significantly; however, the effect of *wtr1* disruption was relatively weak when grown on sawdust medium for 13 d. Also, *vp3* transcript accumulation was significantly reduced in *gat1* and *chd1* disruptants, but not in *wtr1* and *pex1* disruptants.

4. Discussion

This study demonstrates that the mutations in the *gat1* gene encoding a putative zinc finger protein that is conserved among Agaricomycetes cause dominant defects in wood lignin degradation

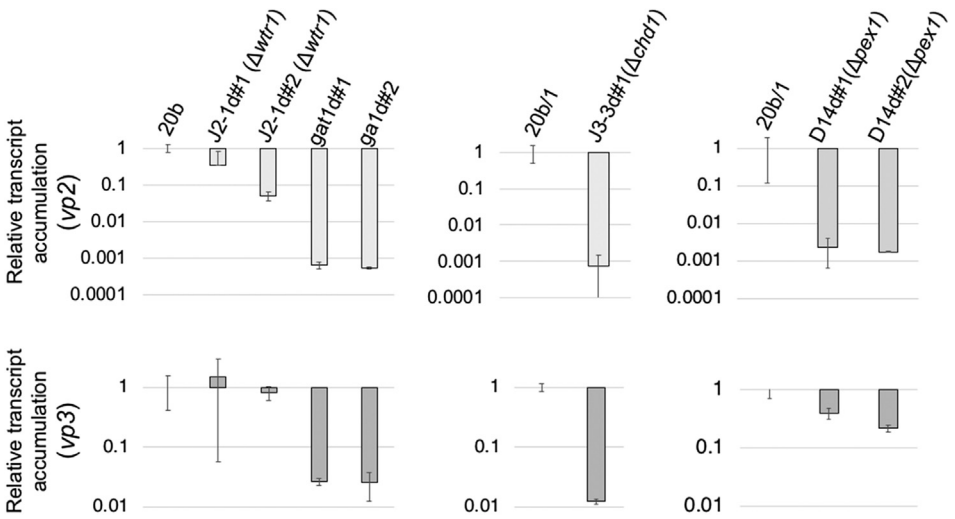

Fig. 4. Quantitative RT-PCR analysis to examine the effects of *wtr1*, *gat1*, *chd1* and *pex1* disruptions on transcript accumulation of *vp2* and *vp3*. Error bars represent standard deviation (n = 2). Transcription levels were normalized to those of the *β-tubulin* gene, in order to calculate the relative transcript accumulation in each gene disruptant relative to its corresponding parental strain.

and fruiting of *P. ostreatus*. Even though Gat1 harbors a zinc finger motif, the possibility that Gat1 is not a DNA-binding transcription factor cannot be excluded because the DNA-binding capacity of Gat1 has not been demonstrated in this study. Furthermore, Gat1 does not seem to contain any typical nuclear localization signal. Therefore, analysis of subcellular localization and identification of proteins associated with Gat1 should be carried out in future to determine if Gat1 functions as a nuclear transcription factor.

The *gat1* gene was previously reported to be involved in fruiting body development in *S. commune*. Ohm et al. (2011) described that Δ*gat1* × Δ*gat1* dikaryon forms more but smaller fruiting bodies than a control wild-type dikaryon in *S. commune*; however, the opposite result was obtained in this study: Δ*gat1*×*gat1*$^{+}$ dikaryon is defective in fruiting body development in *P. ostreatus*. Therefore, it would be interesting to investigate/compare the role(s) of Gat1 in fruiting body development in *S. commune* and *P. ostreatus*, although it remains unclear whether the effects of the *gat1* mutations are direct or indirect in each of these species.

Considering the opposite effects of *gat1* mutations on fruiting development in *P. ostreatus* and *S. commune*, it is likely that the effect of *gat1* mutations on the ligninolytic activity might also differ among white-rot fungi. However, it is challenging to disrupt genes homologous to *gat1* in various white-rot fungi because the frequency of homologous recombination is generally very low (Salame et al., 2012). Therefore, ectopic expression of the *gat1-1* dominant mutant gene may be a more realistic approach to cause malfunction of Gat1-mediated mechanisms in various white-rot fungi; however, it has not been examined whether *gat1* mutations also cause dominant effects in other agaricomycete including *S. commune* (Ohm et al., 2011). Weber et al. (2005) has shown examples of ectopic expression of dominant mutant genes in *S. commune*. This methodology is applicable to some white-rot fungi in which genetic transformation is available, such as *Trametes versicolor* and *Phanerochaete sordida* (Kim et al., 2002; Yamagishi et al., 2007), if *gat1* mutations also cause dominant effects in these species. *S. commune* was recently suggested to be an intermediate between white-rot and brown-rot fungi by the comparative genomic study (Riley et al., 2014): although any known lignin-modifying enzyme genes do not present in the genome of *S. commune*, it has limited lignin-degrading capacity (Schmidt and Liese, 1980; Kersten et al., 2014). Therefore, comparative analysis of *gat1* mutants among *S. commune* and various white-rot fungi might provide new insights into the evolution of fungal wood decay and the molecular mechanisms conferring the diversity of strategies for wood decomposition.

We have shown that *vp2* and *vp3* transcripts accumulate predominantly when *P. ostreatus* PC9, 20b, and 20b/1, are grown on the beech wood sawdust media for 13 d, whereas the transcriptional expression of the both genes is inactivated by *gat1* disruption (Fig. 4). Salame et al. (2014) and Nakazawa et al. (2017b) showed that *vp2* single-gene disruption does not decrease the ligninolytic activity significantly. Therefore, effects of *vp2*/*vp3* double-gene disruption and *vp3* single-gene disruption on the ligninolytic activity should be analyzed to examine whether VP2 and VP3 (or only VP3) are responsible for degradation of wood lignin contained in the beech wood sawdust medium. However, it has also been shown that *pex1* and *wtr1* disruptions caused significant decrease in lignin degradation as well as in accumulation of *vp2* transcripts, but not *vp3* (Nakazawa et al., 2017b, Fig. 4), suggesting that *vp3* single-gene and *vp2*/*vp3* double-gene disruptions do not cause defects in the ability to degrade wood lignin significantly. Considering the fact that RNA-seq analyses were performed using only 13-day culture samples in this study (Table S4), it is quite likely that *vp*/*mnp* gene(s) other than *vp2* and *vp3* may be active at the transcriptional level at other culture periods. In the white-rot fungus *Obba rivulosa*, transcript accumulation of some *mnp* genes were shown to significantly change depending on the culture period (Marinović et al., 2018). Therefore, temporal changes in all *vp*/*mnp* genes at transcriptional (and protein) level should also be investigated in *P. ostreatus* in order to examine which *vp*/*mnp* genes are responsible/essential for wood lignin degradation. However, it is also possible that proteins other than VPs/MnPs are responsible for degradation of wood lignin contained in the beech wood sawdust medium.

In conclusion, mutations in a gene encoding a putative transcription factor, Gat1, cause significant defects in wood lignin degradation and fruiting body development of *P. ostreatus*, although the mechanism of action of Gat1 remains unclear. Assuming that Gat1 is a DNA-binding transcription factor, identification of genes whose transcription is directly controlled by Gat1 based on the results of comparative transcriptome analysis between *gat1* disruptants and the parental strain would allow us to comprehensively understand how Gat1 is involved in the ligninolytic system of *P. ostreatus*.

Acknowledgements

This work was supported in part by the Institute for Fermentation, Osaka [(no grant number) to T.N.], the Noda Institute for Scientific Research [(no grant number) to T.N.], the Hokuto Foundation for Bioscience [(no grant number) to T.N.], and JSPS KAKENHI [16K18729 to T.N.]. We would like to thank Prof. Antonio Gerardo Pisabarro (Public University of Navarra, Spain) for providing *P. ostreatus* strain PC9, and Prof. Yitzhak Hadar (Hebrew University of Jerusalem, Israel) for providing *P. ostreatus* strain 20b.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2018.12.007.

References

Alvarez, J.M., Canessa, P., Mancilla, R.A., Polanco, R., Santibáñez, P.A., Vicuña, R., 2009. Expression of genes encoding laccase and manganese-dependent peroxidase in the fungus *Ceriporiopsis subvermispora* is mediated by an ACE1-like copper-fist transcription factor. Fungal Genet. Biol. 289, 104–111.

Arantes, V., Goodell, B., 2014. Current understanding of brown-rot fungal biodegradation mechanisms: a review. In: Schultz, T.P., Goodell, B., Nicholas, D.D. (Eds.), Deterioration and Protection of Sustainable Biomaterials. ACS Publications, Washington, pp. 3–21.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519–546.

Feldman, D., Kowbel, D.J., Glass, N.L., Yarden, O., Hadar, Y., 2017. A role for small secreted proteins (SSPs) in a saprophytic fungal lifestyle: ligninolytic enzyme regulation in *Pleurotus ostreatus*. Sci. Rep. 7, 14553.

Fernandez-Fueyo, E., Ruiz-Duenas, F.J., Ferreira, P., Floudas, D., Hibbett, D.S., Canessa, P., Larrondo, L.F., James, T.Y., Seelenfreund, D., Lobos, S., Polanco, R., Tello, M., Honda, Y., Watanabe, T., San, R.J., Kubicek, C.P., Schmoll, M., Gaskell, J., Hammel, K.E., St John, F.J., Vanden Wymelenberg, A., Sabat, G., Splinter Bondurant, S., Syed, K., Yadav, J.S., Doddapaneni, H., Subramanian, V., Lavin, J.L., Oguiza, J.A., Perez, G., Pisabarro, A.G., Ramirez, L., Santoyo, F., Master, E., Coutinho, P.M., Henrissat, B., Lombard, V., Magnuson, J.K., Kues, U., Hori, C., Igarashi, K., Samejima, M., Held, B.W., Barry, K.W., Labutti, K.M., Lapidus, A., Lindquist, E.A., Lucas, S.M., Riley, R., Salamov, A.A., Hoffmeister, D., Schwenk, D., Hadar, Y., Yarden, O., de Vries, R.P., Wiebenga, A., Stenlid, J., Eastwood, D., Grigoriev, I.V., Berka, R.M., Blanchette, R.A., Kersten, P., Martinez, A.T., Vicuna, R., Cullen, D., 2012. Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc. Natl. Acad. Sci. 109, 5458–5463.

Fernàndez-Fueyo, E., Ruiz-Dueñas, F.J., Martínez, A.T., 2014. Engineering a fungal peroxidase that degrades lignin at very acidic pH. Biotechnol. Biofuels 7, 114.

Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Lukasik, R., 2010. Hemicellulose for fuel ethanol: a review. Biores. Technol. 101, 4775–4800.

Hori, C., Ishida, T., Igarashi, K., Samejima, M., Suzuki, H., Master, E., Ferreira, P., Ruiz-Dueñas, F.J., Held, B., Canessa, P., Larrondo, L.F., Schmoll, M., Druzhinina, I.S., Kubicek, C.P., Gaskell, J.A., Kersten, P., Jphn, F.S., Glasner, J., Sabat, G., BonDurant, S.S., Syed, K., Yadav, J., Mgbeahuruike, A.C., Kovalchuk, A., Asiegbu, F.O., Lackner, G., Hoffmeister, D., Rencoret, J., Gutiérrez, A., Sun, H., Lindquist, E., Barry, K., Riley, R., Grigoriev, I.V., Henrissat, B., Kües, U., Berka, R.M., Martínez, A.T., Covert, S.F., Blanchette, R.A., Cullen, D., 2014. Analysis of the *Phlebiopsis gigantean* genome, transcriptome and secretome provides insight into its pioneer colonization strategies of wood. PLoS Genet. 10, e1004759.

Inada, K., Morimoto, Y., Arima, T., Murata, Y., Kamada, T., 2001. The *clp1* gene of the mushroom *Coprinus cinereus* is essential for *A*-regulated sexual development. Genetics 157, 133–140.

Irie, T., Honda, Y., Watanabe, T., Kuwahara, M., 2001. Homologous expression of recombinant manganese peroxidase genes in ligninolytic fungus *Pleurotus ostreatus*. Appl. Microbiol. Biotechol. 55, 566–570.

Jin, L., Nicholas, D.D., Kirk, T.K., 1990. Mineralization of the methoxyl carbon of isolated lignin by brown-rot fungi under solid substrate conditions. Wood Sci. Technol. 24, 263–276.

Kamitsuji, H., Honda, Y., Watanabe, T., Kuwahara, M., 2004. Production and induction of manganese peroxidase isozymes in a white-rot fungus *Pleurotus ostreatus*. Appl. Microbiol. Biotechnol. 65, 287–294.

Kamitsuji, H., Honda, Y., Watanabe, T., Kuwaraha, M., 2005. Mn^{2+} is dispensable for the production of active MnP2 by *Pleurotus ostreatus*. Biochem. Biophys. Res. Commun. 327, 871–876.

Kersten, P., Cullen, D., 2014. Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes. Fungal Genet. Biol. 72, 124–130.

Kim, K., Leem, Y., Kim, K., Kim, K., Choi, H.T., 2002. Transformation of the medicinal basidiomycete *Tramacetes versicolor* to hygromycin B resistance by restriction enzyme mediated integration. FEMS Microbiol. Lett. 209, 273–276.

Knop, D., Yarden, O., Hadar, Y., 2015. The ligninolytic peroxidases in the genus *Pleurotus*: divergence in activities, expression, and potential applications. Appl. Microbiol. Biotechnol. 99, 1025–1038.

Kolpak, F.J., Blackwell, J., 1976. Determination of the structure of cellulose II. Macromolecules 9, 273–278.

Kudla, B., Caddick, M.X., Langdon, T., Arst Jr., H.N., 1990. The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J. 9, 1355–1364.

Marinović, M., Aguilar-Pontes, M.V., Zhou, M., Miettinen, O., DeVries, R., Mäkelä, M.R., Hildén, K., 2018. Temporal transcriptome analysis of the white-rot fungus *Obba rivulosa* shows expression of a constitutive set of plant cell wall degradation targeted genes during on solid spruce wood. Fungal Genet. Biol. 112, 47–54.

Marmeisse, R., Gay, G., Debaud, J.C., Casselton, L.A., 1992. Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. Curr. Genet. 22, 41–45.

Monahan, B.J., Askin, M.C., Hynes, M.J., Davis, M.A., 2006. Differential expression of *Aspergillus nidulans* ammonium permease genes is regulated by GATA transcription factor AreA. Eukaryot. Cell 5, 226–237.

Nakazawa, T., Honda, Y., 2015. Absence of a gene encoding cytosine deaminase in the genome of the agaricomycete *Coprinopsis cinerea* enables simple marker recycling through 5-fluorocytosine counterselection. FEMS Microbiol. Lett. 362, 123.

Nakazawa, T., Tsuzuki, M., Irie, T., Sakamoto, M., Honda, Y., 2016. Marker recycling via 5-fluoroorotic acid and 5-fluorocytosine counter-selection in the white-rot agaricomycete *Pleurotus ostreatus*. Fung. Biol. 120, 1146–1155.

Nakazawa, T., Izuno, A., Kodera, R., Miyazaki, Y., Sakamoto, M., Isagi, Y., Honda, Y., 2017a. Identification of two mutations that cause defects in the ligninolytic system through an efficient forward genetics in the white-rot agaricomycete *Pleurotus ostreatus*. Environ. Microbiol. 19, 261–272.

Nakazawa, T., Izuno, A., Horii, M., Kodera, R., Nishimura, H., Hirayama, Y., Tsunematsu, Y., Miyazaki, Y., Awano, T., Muraguchi, H., Watanabe, K., Sakamoto, M., Takabe, K., Watanabe, T., Isagi, Y., Honda, Y., 2017b. Effects of *pex1* disruption on wood lignin biodegradation, fruiting development and the utilization of carbon sources in the white-rot Agaricomycete *Pleurotus ostreatus* and non-wood decaying *Coprinopsis cinerea*. Fungal Genet. Biol. 109, 7–15.

Nishimura, H., Kamiya, A., Nagata, T., Katahira, M., Watanabe, T., 2018. Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls. Sci. Rep. 8, 6538.

Ohm, R.A., deJong, J.F., deBekker, C., Wösten, H.A., Lugones, L.G., 2011. Transcription factor genes of *Schizophyllum commune* involved in regulation of mushroom formation. Mol. Microbiol. 81, 1433–1445.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pickard, M.A., Vandertol, H., Roman, R., Vazquez-Duhalt, R., 1999. High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. Can. J. Microbiol. 45, 627–631.

Pollegioni, L., Tonin, F., Rosini, E., 2015. Lignin-degrading enzymes. FEBS J. 282, 1190–1213.

Rao, P.S., Niederpruem, D.J., 1969. Carbohydrate metabolism during morphogenesis of *Coprinus lagopus* (sensu Buller). J. Bacteriol. 100, 1222–1228.

Riley, R., Salamov, A.A., Brown, D.W., Nagy, L.G., Floudas, D., Held, B.W., Levasseur, A., Lombard, V., Morin, E., Otillar, R., Lindquist, E.A., Sun, H., LaButti, K.M., Schmutz, J., Jabbour, D., Luo, H., Baker, S.E., Pisabarro, A.G., Walton, J.D., Blanchette, R.A., Henrissant, B., Martin, F., Cullen, D., Hibbett, D.S., Grigoriev, I.V., 2014. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm, for wood decay fungi. Proc. Natl. Acad. Sci. 111, 9923–9928.

Ritter, G.J., Seborg, R.M., Mitchell, R.L., 1932. Factors affecting quantitative determination of lignin by 72 per cent sulfuric acid method. Ind. Eng. Chem. Anal. Ed. 4, 202–204.

Rytioja, J., Hildén, K., Yuzon, J., Hatakka, A., DeVries, R.P., Mäkelä, M.R., 2014. Plant-polysaccharide-degrading enzymes from basidiomycetes. Microbiol. Mol. Biol. Rev. 78, 614–649.

Sakamoto, T., Kitaura, H., Minami, M., Honda, Y., Watanabe, T., Ueda, A., Suzuki, K., Irie, T., 2010. Transcriptional effect of a calmodulin inhibitor, W-7, on the ligninolytic enzyme genes in *Phanerochaete chrysosporium*. Curr. Genet. 56, 401–410.

Salame, T.M., Yarden, O., Hadar, Y., 2010. *Pleurotus ostreatus* manganese-dependent peroxidase silencing impairs decolourization of Orange II. Microbiol. Biotechnol. 3, 93–106.

Salame, T.M., Knop, D., Tal, D., Levinson, D., Yarden, O., Hadar, Y., 2012. Predominance of a versatile-peroxidase-encoding gene, *mnp4*, as demonstrated by gene replacement via a gene targeting system for *Pleurotus ostreatus*. Appl. Environ. Microbiol. 78, 5341–5352.

Salame, T.M., Knop, D., Levinson, D., Yarden, O., Hadar, Y., 2013. Redundancy among manganese peroxidases in *Pleurotus ostreatus*. Appl. Environ. Microbiol. 79, 2405–2415.

Salame, T.M., Knop, D., Levinson, D., Mabjeesh, S.J., Yarden, O., Hadar, Y., 2014. Inactivation of a *Pleurotus ostreatus* versatile peroxidase-encoding gene (*mnp2*) results in reduced lignin degradation. Environ. Microbiol. 16, 265–277.

Schmidt, O., Liese, W., 1980. Variability of wood degrading enzymes of *Schizophyllum commune*. Holzforschung 34, 67–72.

Suetomi, T., Sakamoto, T., Tokunaga, Y., Kameyama, T., Honda, Y., Kamitsuji, H., Izumitsu, K., Suzuki, K., Irie, T., 2015. Effects of calmodulin on expression of lignin-modifying enzymes in *Pleurotus ostreatus*. Curr. Genet. 61, 127–140.

Toyokawa, C., Shobu, M., Tsukamoto, R., Okamura, S., Honda, Y., Kamitsuji, H., Izumitsu, K., Suzuki, K., Irie, T., 2016. Effects of overexpression of *PKAc* genes on expression of lignin-modifying enzymes by *Pleurotus ostreatus*. Biosci. Biotechnol. Biochem. 80, 1759–1767.

Tsukihara, T., Honda, Y., Sakai, R., Watanabe, T., Watanabe, T., 2006. Exclusive overproduction of recombinant versatile peroxidase MnP2 by genetically modified white rot fungus, *Pleurotus ostreatus*. J. Biotechnol. 126, 431–439.

Tsukihara, T., Honda, Y., Sakai, R., Watanabe, T., Watanabe, T., 2008. Mechanism for oxidation of high-molecular-weight substrates by a fungal versatile peroxidase, MnP2. Appl. Environ. Microbiol. 74, 2873–2881.

Vyas, B.R.W., Molitoris, H.P., 1995. Involvement of an extracellular H_2O_2-dependent ligninolytic activity of the white rot fungus *Pleurotus ostreatus* in the decolourization of Remazol Brilliant Blue R. Appl. Environ. Microbiol. 61, 3919–3927.

Watanabe, T., Koshijima, T., 1988. Evidence for an ester linkage between lignin and glucuronic acid in lignin-carbohydrate complex by DDQ-oxidation. Agric. Biol. Chem. 52, 2953–2955.

Watanabe, T., Ohnishi, J., Yamasaki, Y., Kaizu, S., Koshijima, T., 1989. Binding-site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes by DDQ-oxidation. Agric. Biol. Chem. 53, 2233–2253.

Weber, M., Salo, V., Uuskallio, M., Raudaskoski, M., 2005. Ectopic expression of a constitutively active Cdc42 small GTPase alters the morphology of haploid and dikaryotic hyphae in the filamentous Agaricomycete *Schizophyllum commune*. Fungal Genet. Biol. 42, 624–637.

Yamagishi, K., Kimura, T., Oita, S., Sugiura, T., Hirai, H., 2007. Transformation by complementation of a uracil auxotroph of the hyper lignin-degrading basidiomycete *Phanerochaete sordida* YK-624. Appl. Microbiol. Biotechnol. 76, 1079–1091.

Zhang, X., Yang, W., Blasiak, W., 2011. Modeling study of woody biomass: interactions of cellulose, hemicellulose, and lignin. Energy Fuels 25, 4786–4795.

Zhang, N., Li, S., Xiong, L., Hong, Y., Chen, Y., 2015. Cellulose-hemicellulose interaction in wood secondary cell-wall. Model. Simulat. Mater. Sci. Eng. 23, 085010.